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Receptor-Dependent and Tyrosine Phosphatase-Mediated Inhibition of GSK3 Regulates Cell Fate Choice

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Summary

Asymmetric body axis formation is central to metazoan development. *Dictyostelium* establishes an anterior/posterior axis utilizing seven-transmembrane cAMP morphogen receptors (CARs) and GSK3-mediated signal transductions that has a parallel with metazoan Wnt/Frizzled-GSK3 pathways. In *Dictyostelium*, GSK3 promotes posterior cell patterning but inhibits anterior cell differentiation. Tyrosine kinase ZAK1 mediates GSK3 activation. We now show that CAR4 regulates a tyrosine phosphatase that inhibits GSK3 activity. We have also identified essential phosphotyrosines in GSK3, confirmed their role in activated/deactivated regulation and cell fate decisions, and relate them to the predicted 3D structure of GSK3β. CARs differentially regulate GSK3 activity by selectively activating a tyrosine phosphatase or kinase for pattern formation. The findings may provide a comparative understanding of CAR-GSK3 and Wnt/Frizzled-GSK3 pathways.

Introduction

Morphogen signaling is essential for cell fate specification and pattern formation of multicellular organisms. In the canonical Wnt pathway, signaling antagonizes the activity of the GSK3 protein kinase (Kim and Kimmel, 2000; Ferkey and Kimelman, 2000; McEwen and Peifer, 2000; Ali et al., 2001; Woodgett, 2001; Cohen and Frame, 2001). While active GSK3 will phosphorylate the transcriptional cofactor β-catenin to direct its destabilization, Wnt signaling is functionally inhibitory to GSK3 and thereby stabilizes β-catenin to effect transcriptional regulation (Wodarz and Nusse, 1998; Kim and Kimmel, 2000; Ferkey and Kimelman, 2000; McEwen and Peifer, 2000). Inhibited or active GSK3 will alternatively establish dorsal/ventral patterns in vertebrates, posterior/anterior segment polarity in *Drosophila*, mesoderm/endoderm cell fates in *C. elegans*, and anterior/posterior cell fates in *Dictyostelium*. GSK3 thus serves as an effective developmental switch, with GSK3 regulation being part of an integrated circuit that processes upstream signals in various cellular contexts (Kim and Kimmel, 2000; Ferkey and Kimelman, 2000; McEwen and Peifer, 2000). While inhibition of constitutively active GSK3 had been the paradigm, it is now appreciated that GSK3 regulation can also involve activating mechanisms, scaffolding complexes, and the differential recognition of target substrates (Wodarz and Nusse, 1998; Kim and Kimmel, 2000; Ferkey and Kimelman, 2000; McEwen and Peifer, 2000; Woodgett, 2001; Cohen and Frame, 2001).

In *Dictyostelium*, secreted cAMP serves as a morphogen to direct cell fate specification by stimulating the family of seven-transmembrane, cell surface cAMP receptors (CARs). The body axis of *Dictyostelium* is defined by anterior prestalk cells and posterior prespore cells (Aubry and Firtel, 1999; Kay, 2000; Kim and Kimmel, 2000). GSK3 is required for prespore/spore cell fate specification but is antagonistic to prestalk/stalk patterning, and body axis asymmetry is similarly regulated by CAR3 (Harwood et al. 1995; Plyte et al. 1999) as well as by the tyrosine kinase ZAK1 (Kim et al. 1999); we had previously demonstrated that CAR3 is required to activate ZAK1, that CAR3 and ZAK1 are required to activate GSK3, and that ZAK1 can directly phosphorylate and activate both *Dictyostelium* and mammalian GSK3 in vitro (Kim et al. 1999). We had thus suggested that specific tyrosine phosphorylation (see Kim and Kimmel, 2001; Woodgett, 2001) was potentially a universal mechanism for GSK3 activation and the regulation of cell fate choice (Kim et al. 1999; Kim and Kimmel, 2000).

We now report a cAMP-mediated and protein tyrosine phosphatase-dependent pathway in *Dictyostelium* that is antagonistic to GSK3 function and confirm in vivo phosphorylation/dephosphorylation modes for GSK3 regulation. Signaling through the receptor subtype CAR4 regulates the protein tyrosine phosphatase to direct the deactivation of GSK3. We have also demonstrated that two tyrosine residues within the activation loop of GSK3 are targets for phosphorylation and that their substitution to phenylanine severely compromises kinase activity and cAMP-mediated activation. The data may provide comparative understandings of GSK3-mediated signaling among diverse multicellular organisms and suggest that tyrosine phosphorylation/dephosphorylation may complement other regulatory mechanisms for activation/deactivation of GSK3 and Wnt-mediated regulation of cell fate determination and tumorigenesis.

Results and Discussion

CAR4 Signaling Inhibits GSK3 Activity to Establish Anterior Cell Fate Choice

Asymmetric anterior/posterior body axis formation in *Dictyostelium* requires cAMP receptor (CAR) signaling for regulation of cell type-specific gene expression and for patterning of anterior (prestalk) and posterior (prespore) cells. CAR4 is preferentially expressed in anterior/prestalk cells, and loss of CAR4 leads to the reduction in anterior gene expression and the concurrent enhanced expression of posterior-specific genes (Louis et al.,

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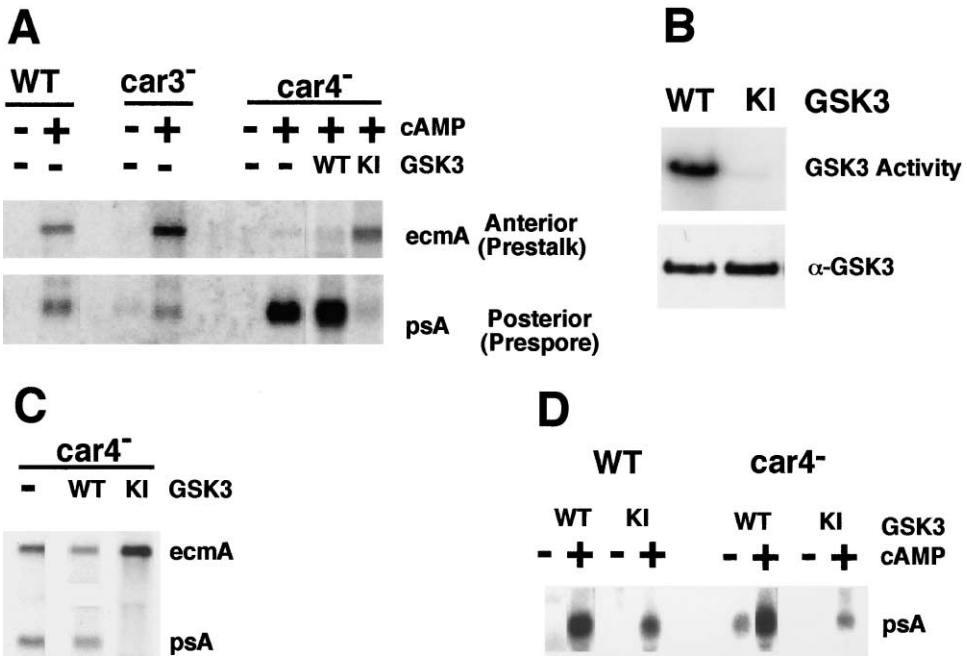

Figure 1. Kinase-Inactive GSK3 Acts as a Dominant Negative to Rescue the *car4* Null Phenotype

(A) A kinase-inactive mutant of GSK3 has dominant-negative developmental effects. Wild-type (WT), *car3* null, and *car4* null cells were differentiated in shaking culture with 50 nM cAMP pulses for 5 hr and then were continuously treated for 6 hr with or without 300 μM cAMP to induce cell-specific gene expression. Northern blot analysis shows the enhanced expression of the anterior prestalk marker *ecmA* in *car3* null cells and reduction of the posterior prespore marker *psA*. Conversely, *psA* is highly upregulated in *car4* null cells, while *ecmA* expression is attenuated. Expression of the K84M, K85M kinase-inactive (KI) GSK3 mutant (see Figure 1B) with the *act15* promoter in the *car4* null background suppressed posterior, prespore gene expression but rescued anterior, prestalk expression. No effect on gene expression was observed using wild-type (WT) GSK3.

(B) The K84M, K85M GSK3 mutant lacks kinase activity. FLAG-tagged wild-type (WT) and FLAG-tagged K84M, K85M (lysine residues involved in ATP binding) kinase-inactive (KI) mutant GSK3 were expressed in *car4* null cells with the *act15* promoter and immunopurified with the α-FLAG antibody M2. GSK3 was normalized with α-GSK3 antibody and specific GSK3 kinase activities measured with MBP (myelin basic protein) as a substrate.

(C) *car4* null controls (−) and *car4* nulls expressing wild-type (WT) or kinase-inactive (KI) forms of GSK3 were developed on solid substrata for 15 hr, and expression of the anterior prestalk marker *ecmA* and the posterior prespore marker *psA* was determined by Northern blot hybridization.

(D) *car4* null and wild-type cells expressing wild-type (WT) or kinase-inactive (KI) forms of GSK3 were developed on solid substrata to the mound stage (12 hr) and harvested. Cells were disaggregated and incubated for 6 hr in the presence of 300 μM cAMP to induce prespore gene expression. Expression of the posterior prespore marker *psA* was determined by Northern blot hybridization.

1994; see Figure 1A). LiCl, an inhibitor of GSK3 (Klein and Melton, 1996; Stambolic et al., 1996), will promote anterior and stalk cell fate differentiation in wild-type cells (Maeda, 1970) and increase anterior gene expression in *car4* nulls (Ginsburg and Kimmel, 1997), suggesting an antagonistic link between CAR4 signaling and GSK3 function. However, it is well recognized that LiCl inhibition is not specific to GSK3. Inositol monophosphatase (IMPase) activity is also sensitive to LiCl, and IMPase regulation of inositol and inositide-phosphate signaling is required for *Dictyostelium* development (Williams et al., 1999). Using a dominant-negative form of GSK3, we have now examined more directly the epistatic relationship of CAR4 signaling to GSK3 function.

Dictyostelium can be induced to differentiate in shaking culture by treatment with exogenous cAMP (Figure 1A). Under these conditions, wild-type cells will express anterior- and posterior-specific genes, respectively, prestalk *ecmA* and prespore *psA*, while unstimulated cells will not express these differentiation markers. CAR4 expression is required for anterior/prestalk differentiation; *car4* null cells were extremely compromised in *ecmA* expression compared with wild-type, and, conversely, they exhibited enhanced differentiation of posterior/prespore *psA* cell patterns (Figure 1A).

If GSK3 were a downstream, negative target of CAR4, then expression of a kinase-inactive (KI) form of *Dictyostelium* GSK3 in *car4* nulls should rescue the prestalk gene expression defect. We mutated GSK3 at two catalytic lysines (see He et al., 1995) that are required for interaction with ATP and showed that it had no detectable kinase activity (Figure 1B). While expression of wild-type GSK3 had no phenotypic effect, expression of the KI-GSK3 in *car4* nulls reversed the mutant phenotype (Figure 1A). Anterior/prestalk gene expression was rescued, whereas GSK3-dependent prespore gene expression was specifically and strongly repressed (Figure 1A). A similar phenotypic rescue was obtained when gene expression patterns of *car4* nulls expressing the KI form of GSK3 were analyzed during normal development on solid substrata (Figure 1C). Finally, we show that KI-GSK3 will equivalently suppress cAMP-dependent prespore gene expression in both *car4* null and wild-type cells that had been dissociated from multicellular aggregates (Figure 1D).

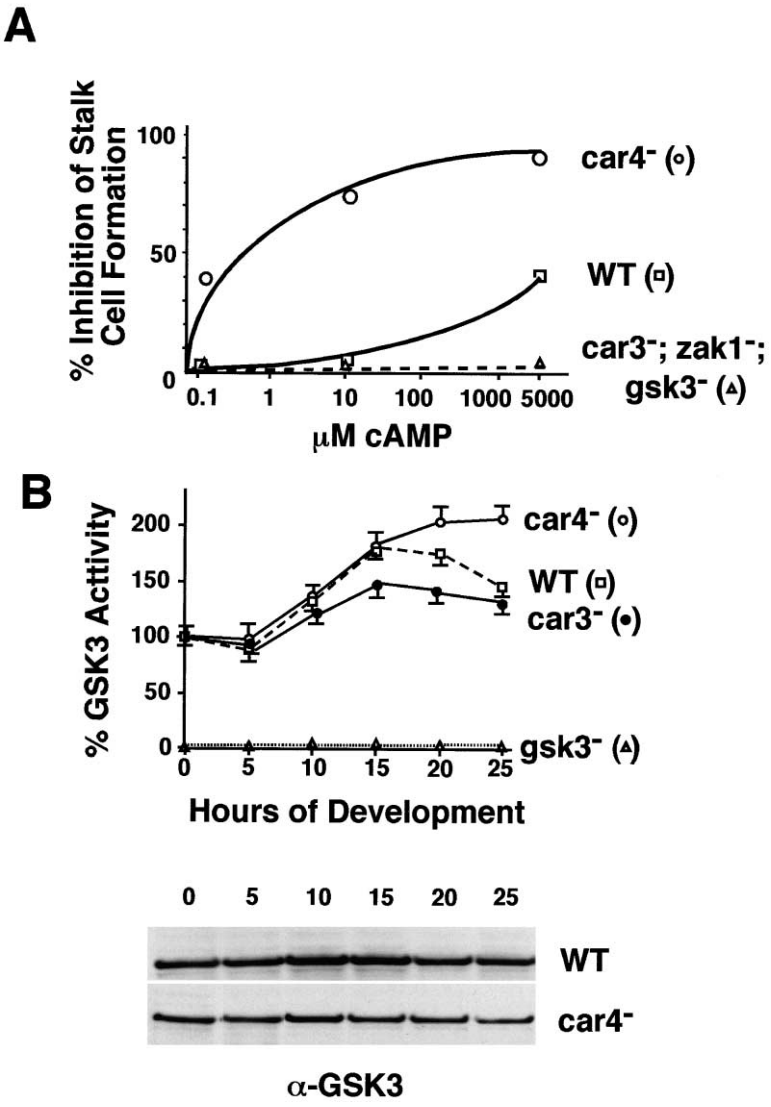

Figure 2. GSK3 Is a Negative Regulatory Target Downstream of CAR4

(A) Stalk cell formation in *car4* nulls is hypersensitive to cAMP. Wild-type (WT), *car4*, *car3*, *zak1*, and *gsk3* null cells were incubated with 5 mM cAMP for 20 hr in monolayer culture (Berks and Kay, 1988) and subsequently incubated with 100 nM DIF-1 at varying concentrations of cAMP, shown in logarithmic scale, for an additional 20 hr. The percentage of vacuolated stalk cells was determined for each assay and expressed as percent inhibition compared with cells treated with DIF-1 in the absence of cAMP (80% stalk cells for WT and 60% stalk cells for *car4* nulls). No inhibition of stalk cell formation by cAMP is observed for *car3*, *zak1*, and *gsk3* null cells. The data represent mean values of two independent experiments.

(B) GSK3 activity is upregulated in *car4* nulls. Whole-cell lysates from wild-type (WT), *car4*, *car3*, and *gsk3* null cells were prepared throughout development and GSK3-specific kinase activities determined by peptide kinase assay. GSK3 was normalized by Western blotting with the α-GSK3 antibody 4G-1E. The data represent mean values of three independent experiments.

Kinase-inactive GSK3 thus behaves as a dominant-negative mutant, under all developmental conditions tested, and rescues the *car4* null phenotype. The data suggest strongly that GSK3 is positioned in a negative circuit downstream of CAR4 and indicate that a receptor inhibitory signaling pathway functions in *Dictyostelium* as a parallel to one mediated by the metazoan Frizzled (Fz) receptors for Wnt ligands (Wodarz and Nusse, 1998; Ferkey and Kimelman, 2000; Kim and Kimmel, 2000).

GSK3 inhibits prestalk/stalk gene expression but activates prespore/spore gene expression, and, as seen in Figure 1A, loss of CAR3 resulted in the expansion of prestalk gene expression at the expense of prespore gene patterning (Harwood et al., 1995; Plyte et al., 1999; Kim et al., 1999), a phenotype opposite that of *car4* nulls. Thus, there appears to be a balance between CAR4-regulated antagonism toward GSK3 and CAR3-regulated GSK3 activation. At the terminal stage of differentiation, elevated GSK3 activity will repress the DIF-1-mediated (see Berks and Kay, 1988) stalk cell differentiation pathway (Harwood et al., 1995; Plyte et al., 1999; Kim et al., 1999). Therefore, if CAR4 serves to inhibit GSK3 activity, cAMP stimulation of CAR4 at the terminal stage of differentiation should suppress GSK3 to potentiate stalk cell formation. Simultaneous activation of GSK3 via CAR3 would inhibit stalk differentiation. In support, stalk cell formation in *car4* nulls was inhibited by a cAMP concentration that is three to four orders of magnitude below that required for inhibition of wild-type cells (Figure 2A). cAMP was unable to suppress DIF-1-mediated stalk cell formation in *car3* or *gsk3* nulls, cells with inherently diminished GSK3 activity (Harwood et al., 1995; Plyte et al., 1999; Kim et al., 1999). These genetic data further affirm that CAR4 organizes an inhibitory circuit that is antagonistic to CAR3-mediated activation of GSK3 and may partially explain the complexity and antagonism of cAMP/DIF-1 signal/response in *Dictyostelium* (Berks and Kay, 1990).

The cell-type specific gene expression patterns caused by loss of CAR4, together with the epistatic relationship of GSK3 and CAR4, led us to investigate whether GSK3 activity itself were specifically upregulated in cells that lack the antagonistic CAR4 pathway. Growing cells exhibit basal GSK3 activity levels. During wild-type development, the specific activity of GSK3 increases ~2-fold, coincident with the onset of anterior/

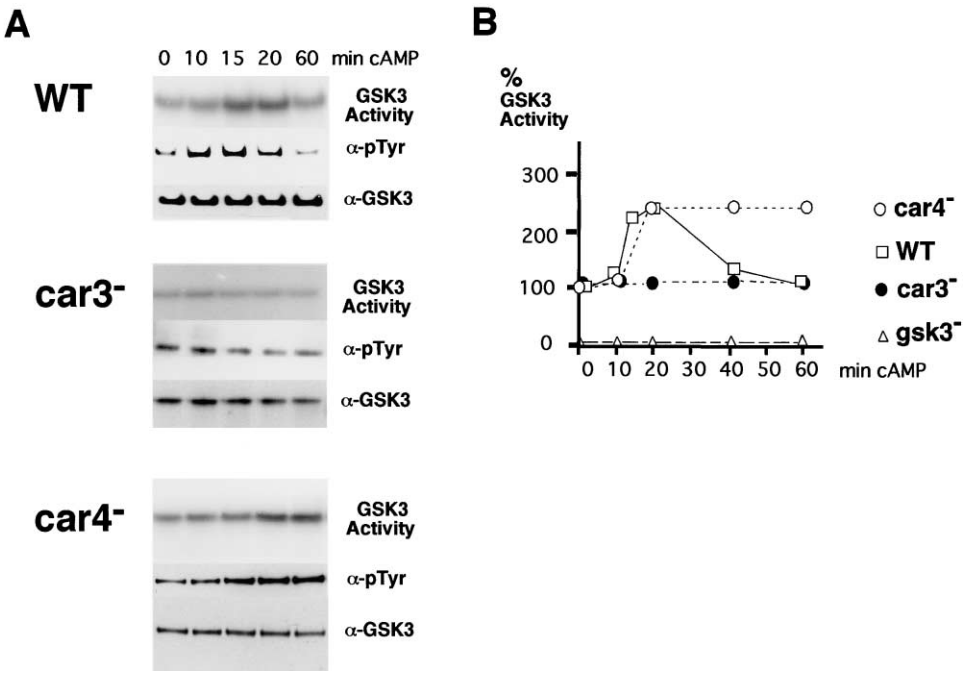

Figure 3. Antagonistic Regulation of GSK3 by Tyrosine Phosphorylation and Dephosphorylation

(A) Differential tyrosine phosphorylation of GSK3 by CAR3 and CAR4 regulates GSK3 activity. GSK3 was fused in-frame with the FLAG peptide and expressed in wild-type (WT), *car3*, and *car4* null cells with the *act15* promoter. Cells were differentiated in shaking culture with 50 nM cAMP pulses for 4 hr and then treated with 300 μM cAMP for the times indicated. Whole-cell lysates were prepared throughout the time course, and GSK3 was immunoprecipitated with α-FLAG antibody M2. GSK3 was eluted by FLAG peptide and normalized by Western blotting with α-GSK3 antibody 4G-1E. The same filters were stripped and reprobed using α-phosphotytrosine antibody 4G10. GSK3-specific activities were measured from purified GSK3 with MBP as a substrate.

(B) Relative changes in GSK3 activity levels following cAMP stimulation in an experiment similar to that of Figure 3A. GSK3-specific activity for each strain at 0 time was set to 100%. GSK3 activity in the *gsk3* null strain was compared with that of wild-type cells at 0 time (see Kim et al. 1999).

posterior cell differentiation (Plyte et al., 1999; Kim et al., 1999; see also Figure 2B). At later developmental stages, GSK3 activity returns to near-basal levels (Plyte et al., 1999; Kim et al., 1999; see also Figure 2B). GSK3 displayed normal activation in the absence of CAR4 but lacked the terminal inhibitory phase (Figure 2B); elevated activity of GSK3 persisted throughout late development. This is consistent with the loss of a CAR4-dependent inhibitory pathway for the regulation of GSK3 activity in *car4* nulls. GSK3 activation during early development requires signaling through CAR3 (Plyte et al., 1999; Kim et al., 1999) and full activation of GSK3 is absent in *car3* nulls (Figure 2B). Thus, CAR4 and CAR3 antagonistically regulate GSK3 at the level of kinase activity to establish cell fate choice.

CAR4 Inhibition of GSK3 Is Mediated by Tyrosine Dephosphorylation

Previous data indicated that activation of GSK3 required phosphorylation by the ZAK1 tyrosine kinase (Kim et al., 1999). We next determined whether the mechanism for CAR4 inhibition of GSK3 involved the regulation in vivo of tyrosine phosphorylation levels. We expressed FLAG-tagged GSK3 in wild-type, *car3* null, and *car4* null cells and monitored the tyrosine phosphorylation state and specific activity of GSK3 following cAMP stimulation of cultured cells; phenotypes of the parental cell lines were not altered by GSK3 expression (see Figure 1). Under these cell culture conditions, stimulation of wild-type cells with cAMP induced a rapid increase in both tyrosine phosphorylation and activation of GSK3 (Figures 3A and 3B). GSK3 activation may be slightly delayed relative to phosphorylation, suggesting a dependent relationship. GSK3 subsequently underwent a rapid dephosphorylation phase, where in-parallel GSK3 activity also returned to basal levels. These data are consistent with phosphorylation/dephosphorylation as the antagonostic mechanism for activation/deactivation of GSK3. In *car3* null cells GSK3 has a basal level of phosphorylation, but cAMP treatment was unable to activate ZAK1 (Kim et al., 1999) or to mediate tyrosine phosphorylation or activation of GSK3 in vivo (Figures 3A and 3B). The basal phsophorylation of GSK3 in *car3* nulls may derive from signaling through the closely related receptor CAR1, whose expression is downregulated during this developmental stage.

In contrast, while normal cAMP-stimulated tyrosine phosphorylation and activation of GSK3 were observed in the absence of CAR4, tyrosine phosphorylation of GSK3 remained elevated and did not decline with time in the *car4* nulls (Figures 3A and 3B). Furthermore, the persistence of the tyrosine phosphorylation was paralleled by a persistence in GSK3 activation throughout the entire course of the experiment. The absence of

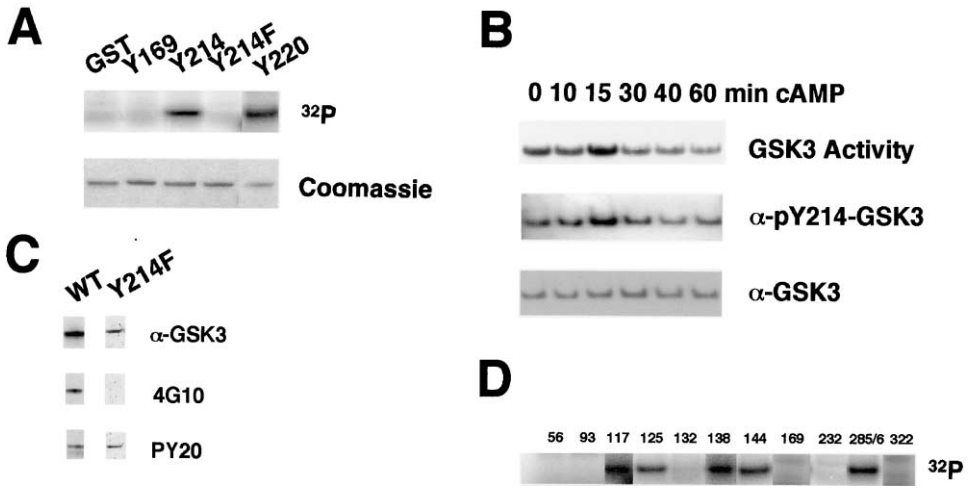

Figure 4. Y214 and Y220 of GSK3 Are In Vivo and In Vitro Phosphorylation Sites

(A) Identification of ZAK1 phosphorylation sites in GSK3. GST was fused in-frame with peptides from *Dictyostelium* GSK3 that contained tyrosine residues plus flanking sequences. Each construct was separately tested in vitro for its capacity as a ZAK1 substrate. Relative levels of expressed protein were assessed by Coomassie blue staining.

(B) cAMP-induced phosphorylation of Y214. GSK3 was fused in-frame with the FLAG peptide and expressed in wild-type cells with the *act15* promoter. Cells were differentiated in shaking culture with 50 nM cAMP pulses for 4 hr and then treated with 300 μM cAMP for the times indicated. Whole-cell lysates were prepared throughout the time course, and GSK3 was immunoprecipitated with α-FLAG antibody M2. GSK3 was eluted by FLAG peptide and normalized by Western blotting with α-GSK3 antibody 4G-1E. The same filters were stripped and reprobed using α-pY216 antibody 5G-2F. GSK3-specific activities were measured from purified GSK3 with MBP as a substrate.

(C) Wild-type (WT) and Y214F GSK3 was fused in-frame with the FLAG peptide and expressed in wild-type cells with the *act15* promoter. Whole-cell lysates were prepared from 12 hr mounds and GSK3 was immunoprecipitated using α-FLAG antibody M2. GSK3 was eluted by FLAG peptide and normalized by western blotting using α-GSK3 antibody 4G-1E. The same filters were stripped and reprobed with α-phosphotyrosine antibodies 4G10 and PY20.

(D) Identification of ZAK1 phosphorylation sites in GSK3. GST was fused in-frame with peptides from *Dictyostelium* GSK3 that contained the specified tyrosine residue. Each construct was separately tested in vitro for its capacity as a ZAK1 substrate. We were unable to obtain a stable GST fusion with a Y159/Y161 peptide.

dephosphorylation and deactivation of GSK3 that is characteristic of wild-type suggests that cAMP-engaged CAR4 downregulates GSK3 activity by directing a specific loss of ZAK1-mediated tyrosine phosphorylation.

Critical Tyrosine Phosphorylation Sites Lie within the Activation Loop of GSK3

Dictyostelium and mammalian GSK3 share 14 (of 16) tyrosine residues within their respective kinase domains. We had previously shown that ZAK1 can phosphorylate and activate both *Dictyostelium* and mammalian GSK3 in vitro (Kim et al., 1999); these tyrosine phosphorylation sites are also the postulated targets for CAR4-mediated dephosphorylation and deactivation of GSK3. It is therefore significant to identify the phosphorylated tyrosines on GSK3 that mediate activation. To this end, we have analyzed tyrosine residues within *Dictyostelium* GSK3 in a series of in vitro and in vivo mapping studies for ZAK1-mediated phosphorylation and confirmed their function by expression of mutant GSK3s.

ZAK1 has very strong substrate specificity, and, despite the numerous tyrosine residues of GST, ZAK1 did not phosphorylate GST in vitro (Figure 4A). GST was fused in-frame with peptides from *Dictyostelium* GSK3 that contained tyrosine residues and flanking sequences. Each construct was separately tested in vitro for its capacity as a ZAK1 substrate. ZAK1 phosphorylation was very specific; for example, constructs with peptides containing tyrosine 214 (Y216 for mammalian GSK3β; see Experimental Procedures for amino acid numbering) and tyrosine 220 (Y222 for mammalian GSK3β) of *Dictyostelium* GSK3 exhibited strong phosphorylation by ZAK1 (Figure 4A), whereas constructs carrying a Y214F mutation that were otherwise identical in sequence to wild-type were completely inactive to phosphorylation by ZAK1 (Figure 4A).

Using an antibody specific for pY214 (pY216 of GSK3β), we examined whether Y214 was phosphorylated in vivo. cAMP induced a rapid increase in Y214-specific phosphorylation in wild-type cells expressing FLAG-GSK3, which was temporally parallel to the activation of GSK3 (Figure 4B). In addition, pY214 was also dephosphorylated synchronously with the GSK3 deactivation phase and return to its basal activity state. However, Y214 is not the only tyrosine of GSK3 subject to phosphorylation. A Y214F mutant of FLAG-GSK3 was expressed in *Dictyostelium* and probed with different α-phosphotyrosine antibodies. While 4G10 (or α-pY214 [see Figure 5B]) recognizes wild-type GSK3, it fails to crossreact strongly with Y214F GSK3, suggesting that 4G10 primarily recognizes pY214 (Figure 4C). However, α-phosphotyrosine-specific antibody PY20 recognizes Y214F GSK3 as well as it recognizes wild-type GSK3, suggesting that there are in vivo tyrosine phosphorylation sites of GSK3 in addition to Y214. Indeed, a GST

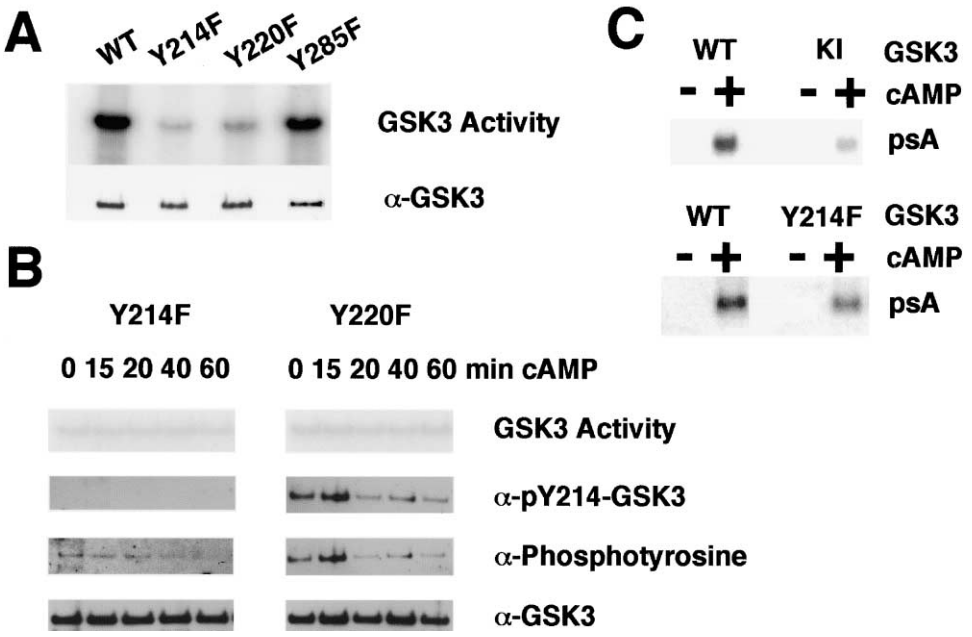

Figure 5. Y214 and Y220 Are Essential for cAMP-Regulated Phosphorylation and Activation of GSK3

(A) Y214F and Y220F mutations of GSK3 have reduced activity. FLAG-tagged wild-type (WT) and Y214F, Y220F, and Y285F mutants of GSK3 were expressed with the *act15* promoter and immunopurified with the α-FLAG antibody M2. GSK3 was normalized with α-GSK3 antibody, and specific GSK3 kinase activities were measured with MBP as a substrate.

(B) Y214F and Y220F GSK3 were fused in-frame with the FLAG peptide and expressed in wild-type cells with the *act15* promoter. Cells were differentiated in shaking culture with 50 nM cAMP pulses for 4 hr and then treated with 300 μM cAMP for the times indicated. Whole-cell lysates were prepared throughout the time course, and GSK3 was immunoprecipitated with α-FLAG antibody M2. GSK3 was eluted by FLAG peptide and normalized by Western blotting with α-GSK3 antibody 4G-1E. The same filters were stripped and reprobed with α-pY216 antibody 5G-2F and with α-phosphotytrosine antibody 4G10. GSK3-specific activities were measured from purified GSK3 with MBP as a substrate.

(C) Wild-type cells expressing wild-type (WT), kinase-inactive (KI), or Y214F forms of GSK3 cells were differentiated in culture with 50 nM cAMP pulses and then continuously treated with or without 300 μM cAMP to induce preporel-specific gene expression. Expression of the prespore marker *psA* was determined by Northern blot hybridization.

fusion with a Y220 peptide of GSK3 served as a very strong substrate for ZAK1 in vitro (Figure 4A). (Additional phosphorylation sites at Y117, Y125, Y138, Y144, and Y285/Y286 in *Dictyostelium* GSK3 that are conserved in mammalian GSK3β were also detected [see Figure 4D].)

Tyrosine Phosphorylation Is Required for GSK3 Activation

To determine whether tyrosines Y214 and Y220 within the activation loop of GSK3 were required for phosphorylation-mediated activation, we expressed wild-type, Y214F, Y220F, and Y285F forms of FLAG-GSK3 in *Dictyostelium* and measured their relative activity levels. Only minimal kinase activity could be detected for the Y214F and Y220F mutant forms, whereas Y285F GSK3 had an equivalent specific activity to wild-type GSK3 (Figure 5A). This establishes a functional association of the Y214 and Y220 sites with GSK3 activity. Y214F and Y220F FLAG-GSK3-expressing cells were challenged with cAMP and kinase activities, and phosphotyrosine levels were monitored. Y214F GSK3 has only minimal basal levels of tyosine phosphorylation and is completely resistant to cAMP-mediated phosphorylation and cAMP-mediated activation. Y220F exhibits basal levels of Y214-specific and total tyrosine phosphorylation, similar to that seen with wild-type GSK3 (see Figure 3A). As with Y214F GSK3, the activity of Y220F GSK3 is insensitive to cAMP stimulation. Rather, we only detect a significant loss of both Y214-specific and total tyrosine phosphorylation that matches temporally the decline in both activity and total tyrosine phosphorylation of wild-type GSK3 (see Figure 3A). Thus, activation of cAMP-dependent tyrosine dephosphorylation is temporally associated with GSK3 deactivation and is correlated with CAR4 function (see Figures 1, 2, and 3).

These data also indicate that Y214 and Y220 are targets for cAMP-regulated phosphorylation and that both pY214 and pY220 are critical elements in the pathway for GSK3 activation. Y214F, unlike the inactivating KI mutant of GSK3, retains a minimal, basal enzymatic activity (see Figures 1B and 5A). However, the inability of Y214F GSK3 to be hyperactivated by cAMP in vivo may indicate that it, like KI GSK3, could serve as a partial dominant negative when expressed in wild-type cells. Indeed, both KI GSK3 and Y214F GSK3 suppressed cAMP/GSK3-dependent expression of the prespore-specific gene *psA* compared with wild-type GSK3 (Figure 5C).

Significantly, tyrosine residues at both 214 and 220 (Y216 and Y222 in mammalian GSK3β), which are essential for full kinase activity, lie within the activation loop of GSK3. Recent resolution of the GSK3β crystal structure (Dajani et al., 2001; ter Haar et al., 2001) may provide novel insight into potential regulatory mechanisms of

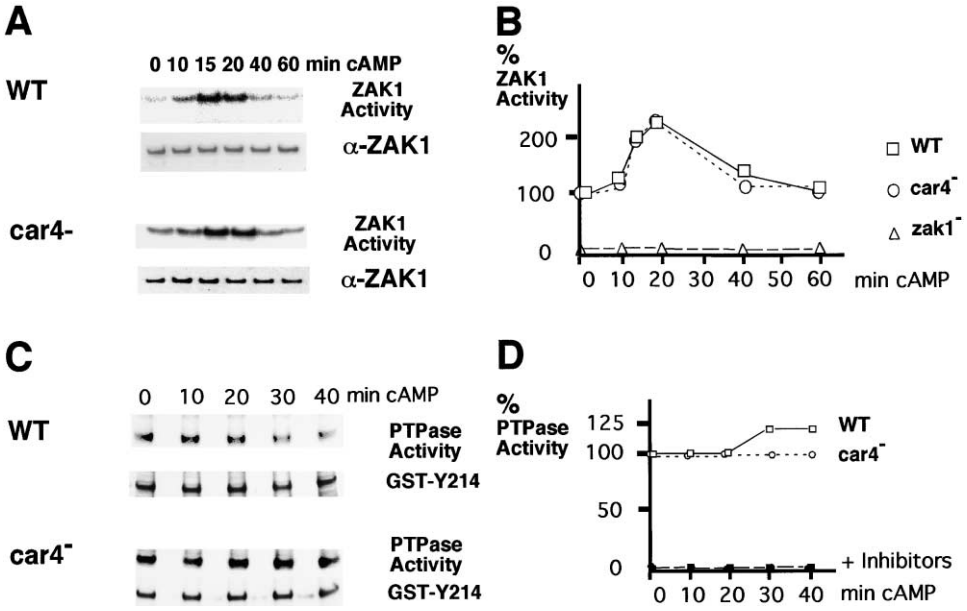

Figure 6. CAR4-Dependent Dephosphorylation of GSK3 Is Mediated by a Protein Tyrosine Phosphatase

(A) ZAK1 is activated transiently in both wild-type and *car4* null cells. Tyrosine kinase ZAK1 was FLAG-tagged and expressed in wild-type and *car4* null cells with the *act15* promoter. Developmentally competent cells were treated with 300 μM cAMP for the times indicated, and whole-cell lysates were prepared. After ZAK1 purification with M2 antibody, ZAK1 levels for each time point were normalized by Western blotting with α-ZAK1 antibody. The ZAK1-specific activities were measured with MBP as a substrate.

(B) Relative changes in ZAK1 activity levels following cAMP stimulation in an experiment similar to that in (A). ZAK1-specific activity for each strain at 0 time was set to 100%. ZAK1 activity in the *zak1* null strain was compared with that of wild-type cells at 0 time (see Kim et al. 1999).

(C) Absence of cAMP-activated GSK3-specific PTPase in *car4* nulls. Developmentally competent cells were treated with 300 μM cAMP for the times indicated, and whole-cell lysates were prepared. Extracts with equivalent protein concentrations were incubated with ^{32}P-Y214 GSK3 peptide in fusion with GST (see Figure 4A). GSK3-specific PTPase activity was measured as a loss of substrate ^{32}P radiolabel, without a concomitant decrease in total substrate (GST-Y214) mass, as determined by Coomassie blue staining.

(D) Relative changes in PTPase activity levels following cAMP stimulation in an experiment similar to that in (C). PTPase activity for each strain at 0 time was set to 100%. The data represent mean values of three independent experiments, ± 4%. Comparative PTPase activity was determined for wild-type cells and *car4* nulls at every time point in the presence of the universal inhibitors sodium vanadate and sodium molybdate.

GSK3 by tyrosine phosphorylation. The activation loop of unphosphorylated GSK3β resembles that of activated (i.e., phosphorylated) ERK2. Phosphotyrosine 185 of ERK2 makes stable ionic interactions with the positively charged, neighboring arginines at positions 189 and 192. These interactions establish an open conformation for the activation loop of ERK2 and thereby facilitate substrate access to the active site. Although unphosphorylated GSK3β exhibits an overall open structural conformation that is similar to activated ERK2, unphosphorylated Y216 of GSK3β (Y214 of *Dictyostelium* GSK3), the tyrosine comparable to Y185 of ERK2, is unable to establish stable interactions with the corresponding arginines R220 and R223. Upon phosphorylation, however, Y216 may adopt an equivalent conformation to the phospho form of ERK2 and, thus, fully facilitate substrate access to the GSK3β active site (ter Haar et al., 2001; Dajani et al., 2001). Similarly, phosphorylation of Y222 in GSK3β (Y220 of *Dictyostelium* GSK3) may also stabilize conformation of the GSK3 activation loop.

Several reports have shown that the tyrosine phosphorylation of GSK3 is critical for kinase activity; mammalian GSK3β purified from muscle tissue is fully active and phosphorylated at Y216 (Hughes et al., 1993), and substitution of Y214 or Y220 in *Dictyostelium* GSK3 is detrimental to kinase activity (Figures 5A and 5B). But, it is also significant that the Y214F (or Y220F) mutant is not completely inactive, as is observed with the K84M, K85M mutant (see Figures 1B, 5A, and 5B), yet both exhibit dominant-negative activities in vivo (Figure 5C).

GSK3β can form an inhibitory homodimer in vitro, with Y216 at the center of the dimer interface (Dajani et al., 2001; Fraser et al., 2002). If inhibitory dimers form in vivo, phosphorylation of Y216 and/or Y222 (Y214 and Y220 in *Dictyostelium)* may disrupt them and serve to activate GSK3. We recognize the potential that phosphorylation of other tyrosines can also induce conformational changes and that cooperative and/or hierarchical phosphomodifications may be required for the maximal developmentally regulated activation of GSK3.

CAR4 Directs Dephosphorylation of GSK3 by Activating a Protein Tyrosine Phosphatase, Not by Inhibiting ZAK1 Kinase

Data suggest that CAR4 mediates an inhibitory pathway that regulates GSK3 tyrosine dephosphorylation and, consequently, the enzymatic activity of GSK3 (Figures 1 and 2). Mechanistically, CAR4 could direct GSK3 dephosphorylation/deactivation by inhibiting ZAK1 kinase activity, in the presence of a constitutively active protein tyrosine phosphatase, or CAR4 could mediate the activation of a GSK3 specific tyrosine phosphatase without

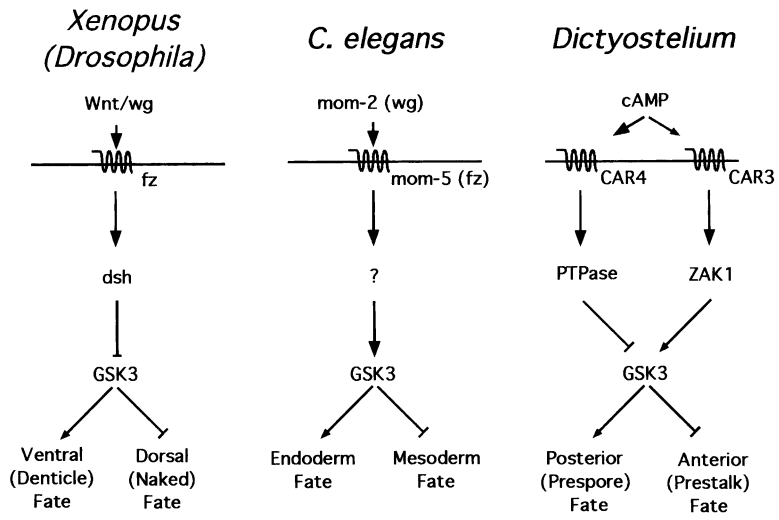

Figure 7. Comparative Cell Fate Choice Pathways in *Xenopus*, *Drosophila*, *C. elegans*, and *Dictyostelium*

Vertebrates, *Drosophila*, *C. elegans*, and *Dictyostelium* share a common developmental dependency on a signaling cascade that is initiated upon stimulation of an Fz domain-containing morphogen receptor that converges at GSK3. In *Xenopus* and *Drosophila*, the morphogen Wnt/Wg initiates an inhibitory signal that requiries dishevelled (dsh) and additional components (data not shown) to functionally downregulate GSK3. This promotes dorsal fate patterning in *Xenopus* and posterior segment polarity (naked cuticle) in *Drosophila* but antagonizes ventral pattern formation in *Xenopus* and anterior segment polarity (denticle formation) in *Drosophila*. Other components, including Axin, mediate the action of active GSK3.

In *C. elegans*, Wnt signaling activates GSK3. The Wnt homolog mom-2 initiates a positive signal cascade from the Fz homolog mom-5 to GSK3. Active GSK3 is required for endoderm formation, while loss of GSK3 function will lead to a mesodermal fate. Molecular intermediates in this pathway have not been identified.

Dictyostelium, in contrast, employs both positive and negative signaling cascades downstream of its morphogen cAMP receptors to regulate the common target GSK3. Stimulation of CAR3 activates GSK3 and promotes prespore fate patterns, whereas CAR4 stimulation will inhibit GSK3 and promote prestalk differentiation. CAR3 activation of GSK3 is mediated by the tyrosine kinase ZAK1, while CAR4 inhibits GSK3 by a tyrosine phosphatase (PTPase)-dependent pathway. Thus, tyrosine phosphorylation/dephosphorylation of GSK3 mediates antagonistic receptor regulation for GSK3 activation/deactivation and cell fate determination.

altering ZAK1 activity. To distinguish between these two pathways, we examined ZAK1 regulation in the absence of CAR4. In wild-type cell cultures, ZAK1 activation by exogenous cAMP was transient (Figures 6A and 6B), effectively paralleling the tyrosine phosphorylation and activation of GSK3 (see Figures 3A and 3B). The absence of CAR4 signaling had no effect on the temporal kinetics of ZAK1 activation by cAMP or on ZAK1 deactivation. CAR4 did not inhibit ZAK1 activation, and CAR4 was not required to inactivate ZAK1 precociously. Therefore, dephosphorylation and deactivation of GSK3 by CAR4 must occur independently of ZAK1 regulation, indicating that dephosphorylation of GSK3 is regulated by a specific CAR4-dependent signaling cascade that activates a protein tyrosine phosphatase, leading to GSK3 deactivation.

We have directly assayed for cAMP/CAR4-mediated protein tyrosine phosphatase (PTPase) activity using a phosphotyrosine GSK3 substrate. Extracts were prepared from wild-type and *car4* null cells at various times following stimulation with cAMP. To determine PTPase activity levels (see Figures 6C and 6D), we incubated extracts with the Y214 peptide of GSK3 fused with GST (see Figure 4A) that had been radiolabeled in vitro with ZAK1. Both extracts had significant basal PTPase. This was seen when PTPase activities were assayed in the presence or absence of the universal inhibitors sodium vanadate and sodium molybdate (Figure 6D).

In wild-type cells, we observed a significant increase in PTPase activity that was only apparent after 20 min of stimulation with cAMP (Figures 6C and 6D). The time course for this cAMP-dependent PTPase activation correlates well with the cAMP-directed loss of phosphotyrosine from GSK3 and the cAMP-directed deactivation of GSK3 in vivo (see Figures 3A, 4B, and 5B). Conversely, no cAMP-activated PTPase was detected in *car4* null cells (Figures 6C and 6D). Thus, we conclude that CAR4 inhibition of GSK3 during development is mediated by the activation of a specific PTPase that serves to dephosphorylate the activating phosphotyrosines within GSK3.

It is significant that activation of the CAR4-regulated PTPase is delayed relative to that of ZAK1 activation but is coordinated with ZAK1 and GSK3 deactivation (see Figure 6A). Thus, the CAR3/ZAK1-dependent phosphorylation/activation of GSK3 anticipates the dephosphorylation inhibitory phase regulated by CAR4 and the PTPase. Effectively, these antagonistic modes for the regulation of GSK3 are temporally isolated. Finally, it should also be emphasized that low-level phosphorylation of Y214 and Y220 of GSK3 persists in both *car3* and *zak1* null cells. This may suggest the potential for signal redundancy mediated by additional CARs and tyrosine kinases.

Conclusion: Pathway Conservation between the cAMP Receptor and Frizzled Families

We argue that the differential regulation of tyrosine phosphorylation of GSK3 by ZAK1 and a PTPase constitutes the core of regulatory machinery on GSK3 in the context of cell fate decision by the seven-transmembrane CARs and suggest that similar mechanistic activities may function during metazoan development. The precise conservation of the essential tyrosines between *Dictyostelium* and mammalian GSK3 supports the potential for a common regulatory network controlling GSK3 activity.

Although CARs and Frizzled (Fz) seven-transmembrane receptors are activated by different ligands, cAMP and Wnt, respectively, and Fz had been considered a strictly metazoan receptor, the resemblance of their signaling pathways converging at GSK3 (Plyte et al. 1999;

Kim et al., 1999) and β-catenin (Grimson et al., 2000) for regulating cell fate decisions suggests an ancient origin for this essential pathway.

In *Drosophila* and vertebrates, canonical Wnt/Fz signaling is inhibitory to GSK3 function, in effective parallel with CAR4. Dominant-negative GSK3 will redirect cell fate choice decision in *Xenopus* (He et al., 1995; Dominguez et al., 1995; Yost et al., 1996), as well as in *Dictyostelium* (Figure 1A). In *Xenopus*, KI-GSK3 is suggested to compete for association of endogenous GSK3 with Axin-based scaffolding complexes (Fraser et al., 2002). Potentially, such complexes are also essential for GSK3 signaling in *Dictyostelium*. CAR3-mediated activation of GSK3 finds equivalence in *C. elegans* (Schlesinger et al., 1999), where the Fz receptor Mom-5 relays an activating Wnt (Mom-2) signal to GSK3 (Figure 7).

It will be very critical to determine whether cell fate decisions in these other systems are also regulated by differential tyrosine phosphorylation of GSK3. Although, to date, *C. elegans* has the only other positive GSK3 cascade yet identified, several mammalian tyrosine kinases, such as Fyn and Pyk2, are suggested to activate GSK3 (Lesort et al., 1999; Hartigan et al., 2001), raising the possibility that tyrosine phosphorylation of GSK3 may mediate antagonism to Wnt signaling to provide additional regulation for differentiation and tumor suppression (Bienz and Clevers, 2000; Kim et al., 1999; Polakis, 2000, Manoukian and Woodgett, 2002) and that an activated PTPase may be functionally equivalent to Wnt signaling to promote tumorigenesis.

Experimental Procedures

Dictyostelium Cell Culture and Differentiation

Dictysotelium discoidium strain JH10 (see Louis et al., 1994), *car3* null cells, and *car4* null cells were grown in axenic medium with or without 100 μg/ml of thymidine, as required (Kim et al., 1999; Ginsburg and Kimmel, 1997). Cells were developed on nitrocellulose filters at 1×10^7 cells per cm^2 in DB buffer (10 mM sodium phosphate [pH 6.4], 2 mM $MgCl_2$, and 0.2 mM $CaCl_2$). Developing *Dictyostelium* was harvested at the given time point, and either protein or RNA was prepared. Cells at mound stage (12 hr) were collected, dissociated into single cells, washed, and resuspended at 1×10^7 cells per milliliter of DB buffer. Cells were treated either with or without 300 μM of cAMP for 6 hr, and RNAs were prepared. To differentiate cells in shaking culture with cAMP, we starved log phase cells for 1 hr at 2×10^7 cells per milliliter of DB. Fifty nanomolar pulses of cAMP for 6 min intervals were applied to the culture for 4 hr, and 300 mM of cAMP was added. Cells were harvested at the given time points of each experiments. For stalk cell differentiation in monolayer culture (Berks and Kay, 1988), cells were plated at 1×10^4 cells/cm^2 with 5 mM cAMP. After 20 hr the cAMP medium was removed and replaced with 100 nM DIF-1 plus varying concentrations of cAMP.

Molecular Manipulation of GSK3 and ZAK1

Kinase-inactive GSK3 was constructed by mutating lysine residues 86 and 87, which are required for ATP binding, to methionine. The oligonucleotide for the FLAG peptide (MDYKDDDDK) was subcloned in-frame with the amino termini of WT, KI, Y241F, and Y220F GSK3 or ZAK1.

GST-GSK3 Peptide Fusion and Phosphorylation Site Mapping

GSK3 peptides were expressed in fusion with GST in *E. coli*. The GST fusions were purified by glutathione affinity chromatography and eluted with 10 mM glutathione. One microgram of each purified GST fusion protein was tested in vitro as a substrate for ZAK1 kinase (Kim et al., 1999). The GSK3 peptides were Y56 (sequences N52–G60), Y93 (sequences Q89–E97), Y169 (sequences R165–L173), Y214 (sequences T210–R218), Y220 (sequences C216–227), Y232 (sequences G228–I236), Y285/Y286 (sequences A281–K290), and Y322 (sequences K318–S326). The published amino acid sequence of *Dictyostelium* GSK3 has a threonine 6-mer, encoded by $(AC^A/_T)_6$, in its N-terminal region (Harwood et al., 1995), a region with no similarity to mammalian GSK3. The *Dictyostelium* GSK3 cDNA used in this study had one less trinucleotide repeat; the encoded protein has only five threonines and is one amino acid shorter in length than the published sequence. The positions of all relevant amino acids in this study are downstream of the threonine region and, thus, are one amino acid position displaced from that published.

Nucleic Acid Analysis

Total RNA blots were as described (Kim et al., 1999). RNAs were monitored for integrity and loading equivalency (5 μg).

Immunological Techniques and Kinase Assays

α-FLAG antibody M2 and FLAG peptide were from Sigma. The GSK3 and ZAK1 kinase assays were described (Kim et al., 1999). Whole-cell lysates of differentiated cells were prepared, and FLAG-GSK3 or FLAG-ZAK1 was purified. Proteins levels were normalized by Western blotting with α-GSK3 (4G-1E from Upstate) or affinity-purified polyclonal α-ZAK1 serum (Kim et al., 1999), and specific kinase activities were measured with 10 μg of MBP (myelin basic protein; Sigma). Changes in ^{32}P radiolabel on the substrates were monitored by PhosphorImager and quantified by ImageQuaNT (Molecular Dynamics). Tyrosine phosphorylation of the GSK3 was also monitored by Western blotting with α-phosphotyrosine antibodies 4G10 and PY20 or with α-phospho-Y216-GSK3-specific monoclonal antibody 5G-2F from Upstate.

GSK3-Specific PTPase Assays

Wild-type and *car4* null cells were pulsed with 50 nM cAMP for 5 hr, stimulated with 300 μM cAMP, and lysed in buffer containing 50 mM LiCl, 50 mM imidazole (pH 7.5), 0.5% NP40, 10% glycerol, 0.01% β-mercaptoethanol, and protease inhibitor cocktail (complete, Mini tablet from Roche). One microgram of the ZAK1-radiolabled Y214 GSK3-GST fusion was incubated for 10 min with 30 μg of extract at 30°C. GSK3-specific PTPase activities were measured with the substrate Y214 peptide of GSK3 fused with GST that had been radiolabeled in vitro with ZAK1 (see Figure 4A). The loss of ^{32}P radiolabel on the substrates was monitored by PhosphorImager and quantified by ImageQuaNT (Molecular Dynamics). Sodium vanadate (2 mM) and sodium molybdate (80 μM) were used as PTPase inhibitors.

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